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Sublethal effects of the insecticidal fusion protein ω-ACTX-Hv1a/GNA on the parasitoid *Eulophus pennicornis* via its host *Lacanobia oleracea*

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ABSTRACT

BACKGROUND: The neurotoxin peptide ω-ACTX-Hv1a, fused to the carrier molecule GNA presents potential for insect control as a biopesticide, being orally toxic to insect pests from different orders. However, thorough evaluation is required to assure its safety towards non-target invertebrates. Effects of this novel biopesticide on the parasitoid *Eulophus pennicornis* via its host *Lacanobia oleracea* are presented.

RESULTS: Hv1a/GNA did not cause mortality when injected or fed to 5th stage *L. oleracea*, but caused up to 39% reduction in mean larval weight ($p<0.05$) and increased developmental time when injected. When fed, GNA, but not Hv1a/GNA, caused ~35% reduction in larval weight, indicating that host quality was not affected by the fusion protein. Although GNA and Hv1a/GNA were internalized by the hosts following ingestion, and thus available to higher trophic levels, no significant changes on rate of *E. pennicornis* parasitism occurred. The number of parasitoid pupae/host, adult emergence and sex ratio were unaffected by GNA- or Hv1a/GNA-treated hosts ($p>0.05$). The fusion protein was degraded by parasitoid larvae, rendering it non-toxic.

CONCLUSION: Hv1a/GNA has negligible effects on the parasitoid, even under worst-case scenarios. This low toxicity to these insects is of interest in terms of biopesticide specificity and safety to non-target organisms.

Keywords: Fusion protein, *Eulophus pennicornis*, *Lacanobia oleracea*, *Galanthus nivalis* agglutinin, ω-ACTX-Hv1a

1 INTRODUCTION

Neurotoxins derived from spider venoms have the potential to effectively target different insect species whilst being innocuous to vertebrates.[1] However, there are major drawbacks on their practical use as topical insecticides, including inability to be absorbed by the insect cuticle, degradation in the environment,[2] and lack of insecticidal activity when delivered orally.[3]

The demonstration that the *Galanthus nivalis* agglutinin (GNA) is able to cross the insect midgut and reach the haemolymph following ingestion[4] opened the possibility of using it as a carrier molecule for insecticidal peptides. For example, the spider venom peptide *Segestria florentina* toxin 1 (SFI1) is structurally similar to other small spider neurotoxins that target voltage-dependent Ca^{2+} channels, causing flaccid paralysis when injected into *Heliothis virescens* larvae, but inactive when injected into mice.[5] As it is orally inactive against insects, Fitches et al.[2] have engineered a fusion protein comprising of the spider venom toxin SFI1 and GNA. The resulting fusion protein presented a high level of oral toxicity to *Lacanobia oleracea,* which was not observed for its components alone. The oral biological activity of the novel protein was due to the GNA transporting the SFI1 peptide to its site of action in the central nervous system (CNS). More recently, Fitches et al.[6] fused the calcium channel blocker ω-ACTX-Hv1a (Hv1a) from the funnel-web spider *Hadronyche versuta* to GNA. Once again, the fusion protein was effective in controlling a lepidopteran pest, *Mamestra brassicae*[6] and the Colorado potato beetle, *Leptinotarsa decemlineata* (unpublished).

Although insecticidal fusion proteins are effective, their use in the field as either a

biopesticide or when expressed in transgenic plants should ideally be compatible with other pest management strategies, including that of biological control. As a consequence, their potential effects on beneficial non-target organisms, such as parasitoids, need to be evaluated.

Previous work has demonstrated that parasitoids respond differently to exposure to GNA alone. For instance, this lectin can have beneficial effects on biological control agents when expressed in transgenic plants. Bell et al.[7] demonstrated that the damage caused by *L oleracea* to transgenic potato plants expressing GNA was further reduced (ca. 21%) when *Eulophus pennicornis* wasps were used for their biological control. However, indirect deleterious effects of GNA in parasitoids, such as decreased lifespan and fecundity as a consequence of reduced host quality, have been reported.[8-10] GNA can also induce direct insecticidal effects when delivered via artificial diet to parasitoid adults,[11-12] affect parasitoid fecundity when administered via dosed hosts,[13] or even present no effects at all when hosts are fed with artificial diets based on transgenic maize or potato expressing GNA.[14] On the other hand, only limited information is currently available on the impacts of these insecticidal fusion proteins against parasitoids.[15]

The present study evaluates the effects of a fusion protein containing GNA and a modified version of Hv1a (K34Q)[16] on *E. pennicornis* Nees (Hymenoptera: Eulophidae), a gregarious ectoparasitoid of the tomato moth *L. oleracea.* Such studies form part of the biosafety assessment, a pre-requisite for the commercial release of biopesticides.

2 EXPERIMENTAL METHODS

2.1 Expression and purification of the recombinant fusion protein Hv1a/GNA

Proteins were produced by heterologous expression in *Pichia pastoris* (SMD1168H strain) carrying sequences encoding GNA or Hv1a/GNA. Fermentations were carried out in Bio Console ADI 1025 (Applikon) fermentors (2 L vessels), with a continuous 50% glycerol feed for 72 h. Supernatants from the cultures were collected by centrifugation after expression. GNA was purified by hydrophobic interaction chromatography on phenyl-sepharose Pharmacia XK16 column. Fractions containing GNA were reloaded onto a size-exclusion column (HiPrep™ 16/60 Sephacryl S-100, GE-Healthcare). Following purification, recombinant proteins were dialyzed, freeze-dried and stored at -20 °C. Supernatants containing his-tagged Hv1a/GNA were diluted in binding buffer (0.02 M Sodium phosphate, 0.4 M NaCl, pH 7.4). Samples were then loaded onto a HisTrap™ (GE Healthcare) column and eluted with binding buffer containing 0.2 M imidazole. After purification, samples were extensively dialyzed in dist. water at 4 °C and freeze-dried.

2.2 Bioassay of Hv1a/GNA with *L. oleracea*

Lacanobia oleracea were derived from a laboratory culture, reared on artificial diet at 25 °C and 16:8 h (L:D).[17] All bioassays with *L. oleracea* were performed using 450 ml transparent plastic cages. Larval stages were determined by measuring the head capsules, as previously described.[17]

Initially, toxicity of Hv1a/GNA was assayed against *L. oleracea* via injection bioassays. Newly moulted 5th stage larvae were anesthetized with CO_2 and injected with 15 µg (in 5 µl PBS) of BSA (n=37 larvae) or Hv1a/GNA (n=35 larvae) on the

ventral side of their abdomen using a Hamiltonr® syringe (model 25F, needle gauge 25). Larval weight and mortality was assessed daily and compared by t-tests and mortality data were assessed by Kaplan-Meyer Survival analysis.

2.3 Exposure of parasitoid larvae to Hv1a/GNA via the tri-trophic interaction: orally dosed host larvae

Effects of the fusion protein Hv1a/GNA on the parasitoid *E. pennicornis* were investigated via the tri-trophic interaction where host larvae were fed the protein so as to mimic exposure in the field, using the method described by Wakefield et al.[15] Fifth instar *L. oleracea* larvae were fed with 5 µl of a 5% sucrose solution containing 50 µg of BSA (control), Hv1a/GNA or GNA, for a minimum of three and a maximum of four consecutive days. Larvae were weighed daily in order to assure that hosts were of comparable quality to parasitoids. After moulting to 6th stage, larvae (n= 36 for BSA, n= 33 for GNA and n= 38 for Hv1a/GNA treatment) were individually exposed to one newly emerged, fecundated female of *E. pennicornis*. Adult female parasitoids were removed after 24 h, freeze-killed and screened for the presence of mature eggs. Parasitized *L. oleracea* larvae were kept until emergence of *E. pennicornis* at 25 ˚C and 16:8 h (L:D). The rates of parasitism, number of *E. pennicornis* pupae/host, sex ratios and parasitoid emergence rates were assessed and compared by one-way ANOVA.

2.4 Exposure of parasitoid larvae to Hv1a/GNA via the tri-trophic interaction: injected host larvae

To ensure exposure of parasitoid larvae to high levels, the recombinant proteins were also delivered to host larvae via injection, so representing a ‘worst-case’ scenario. Fifth instar *L. oleracea* were exposed to fecundated female *E. pennicornis*, in a proportion of two larvae per parasitoid, for up to four days. After this period,

larvae were screened for the presence of parasitoid eggs, anaesthetized with CO_2 and injected with 15 µg of BSA (control, n=34), GNA (n=34) or Hv1a/GNA (n=50), as described above. Host survival, parasitism, number of pupae per host and rate of *E. pennicornis* emergence were recorded and analysed by one-way ANOVA.

2.5 Internalization of GNA and Hv1a/GNA in host larvae

The presence of Hv1a/GNA or GNA in *L. oleracea* haemolymph was verified by immuno-assay using western blotting with anti-GNA as primary antibody and enhanced luminol-based chemiluminescent (ECL), as previously described.[15] As wasp eggs would take on average 2.7 days to hatch,[18] haemolymph was collected four days after hosts had moulted to 6th stage, i.e., after eggs were laid, hatched and parasitoid larvae started feeding on host larvae.

It was not possible to immuno-detect the fusion protein in parasitoid larvae feeding on hosts that were exposed to GNA or fusion proteins by ingestion. Therefore, in order to verify the fate of Hv1a/GNA following ingestion by *E. pennicornis*, parasitized *L. oleracea* larvae were injected with 15 µg of Hv1a/GNA. Parasitoid larvae feeding on injected larvae were then collected and subjected to western blot as described above.

3 RESULTS

3.1 Effects of Hv1a/GNA when injected into *L. oleracea*

Fifth instar *L. oleracea* larvae were injected with recombinant fusion protein. Survival analysis (log-rank) of injected larvae resulted in no significant differences on mortality between treatments (p=0.149). However, a significant reduction in mean weight was observed in Hv1a/GNA-treated larvae from day 2 (Mann-Whitney

p=0.043) to day 10 (p=0.006). After this period, larvae did not present any differences in mean weight from day 11 onwards (p=0.067) (Fig 1). These results also show that there was a decline in larval weight in both treatments from day 12, coinciding with the end of the larval stage and the onset of pupation. Additionally, a significant increase in development time from 5^{th} to 6^{th} stage was observed in the Hv1a/GNA treatment, compared to the control treatment (t-test; BSA n=20, 7.4±1.53 days to moult; Hv1a/GNA n=15, 8.66±1.87 days to moult; p=0.039).

3.2 Effects of Hv1a/GNA on the host *L. oleracea* via ingestion

After ingesting droplets containing Hv1a/GNA or GNA, *L. oleracea* larvae were shown to internalize the proteins, as detected in haemolymph samples by western blot (Fig 2). Even though the fusion protein band at around 25 kDa appears to be fainter than its degradation products, it would still be made available to higher trophic levels, i.e., parasitoid wasps feeding on the haemolymph would also ingest the fusion protein or GNA.

As with the injection bioassays, droplet feeding of the recombinant Hv1a/GNA had no effect on mortality of *L. oleracea* (Kaplan-Meyer survival, p>0.05, data not shown). In contrast to injection bioassays (Fig 1), droplet feeding of Hv1a/GNA did not affect weight of the host larvae, although GNA induced a significant reduction on this parameter (ANOVA, p< 0.05; Fig 3). Although differences in weight of *L. oleracea* larvae were detected for GNA, only host larvae of similar masses were subsequently offered to *E. pennicornis* adult females (ANOVA, p=0.394). However it is acknowledged that GNA may have caused subtle effects on the suitability of these insects as hosts.

3.3 Effects of Hv1a/GNA on parasitoid performance when hosts were dosed orally

The rate of parasitism of *E. pennicornis* on *L. oleracea*, even though slightly higher in the control, did not differ significantly between treatments (Mann-Whitney, p=0.378; Fig 4). Furthermore, no differences were found in the mean number of *E. pennicornis* pupae/host larva (ANOVA; p=0.889) and sex ratio (p=0.570; Table 1). Although non-significant, control adults started emerging 13 days after *L. oleracea* were exposed to parasitoid adult females, whereas the first adult emergence occurred 15 and 16 days after parasitoid exposure to GNA and Hv1a/GNA treatments, respectively. Dissections of parasitoid females that did not oviposit demonstrated that they all carried mature eggs when in contact with *L. oleracea* (data not shown).

These results indicate that Hv1a/GNA does not affect any of the life parameters investigated for the parasitoid *E. pennicornis.* Neither the fusion protein nor GNA were detected in parasitoid larvae feeding on *L. oleracea* hosts that were previously exposed to those proteins (data not shown).

3.4 Effects of Hv1a/GNA on parasitoid performance when hosts were injected

As no effects were detected on parasitoids developing on hosts that were orally exposed to GNA or Hv1a/GNA, *L. oleracea* hosts were injected with 15 µg of BSA, GNA or Hv1a/GNA after they had been parasitized by *E. pennicornis*, representing a 'worst-case scenario' bioassay. Protein injections following parasitism resulted in high *L. oleracea* mortality, particularly in the fusion protein treatment, in which only 4% of the hosts survived. No significant (p>0.05) differences between control and

GNA treatments were found either on the number of *E. pennicornis* pupae, or number of adults emerged, per host (Table 2). Comparisons between these two treatments and the Hv1a/GNA treatment were not made due to the low number of surviving hosts injected with fusion protein.

Even though the injection of Hv1a/GNA yielded low survival rates for both the host and *E. pennicornis*, parasitoid larvae feeding on *L. oleracea* injected with the fusion protein were collected and subjected to immunoassays. Hv1a/GNA was shown to be degraded following ingestion by parasitoid larvae, as the ~25 kDa band corresponding to the intact fusion protein is not seen on the western blot (Fig 5).

4 DISCUSSION AND CONCLUSIONS

The fusion protein Hv1a/GNA is currently being developed as a biopesticide for controlling important lepidopteran and coleopteran pests.[6] However, it is important that this new biopesticide is also compatible with other pest management strategies, including that of biological control. Commonly used neuroactive insecticides such as pyrethroids, organophosphates, carbamates and carbamyltriazole can be highly toxic to parasitoid wasps at field application rates.[19] Furthermore, some insecticides (e.g. malathion, etofenprox and methomyl) can also have strong, sub-lethal negative effects on the foraging behaviour,[20] while others (e.g. chlorpyrifos) can reduce the sex ratio in parasitoid progenies.[21] It is not expected that Hv1a/GNA would have contact toxicity against insects, as it is an orally-active biopesticide and is not absorbed through the cuticle. Other biopesticides, however, might present contact toxic effects against parasitoids. For example, Spinosad causes high acute mortality on adults and pupae of *Bracon nigricans*. The neurotoxic biopesticides emamectin

benzoate and abamectin induce sub-lethal effects on this parasitoid, affecting its biocontrol activity, whereas Bt is relatively safe.[22]

In order to test effects of a fusion protein against beneficial arthropods, a system that mimics a relevant interaction was selected, since *E. pennicornis* is an effective biological control agent against the tomato moth *L. oleracea.*[23] Additionally, a host that would not be negatively affected by the fusion protein via oral exposure was deliberately used, thus reducing potential effects due to host quality, rather than direct toxicity (as suggested in [24]). Injection of Hv1a/GNA (representing a 'worst case' scenario) into fifth stage larvae of *L. oleracea* caused a delay in developmental time and a temporary significant weight reduction. However, after moulting into the sixth stage, these differences were no longer significant . In contrast, when fed to *L. oleracea*, the fusion protein did not cause any measurable detrimental effects on the larvae, presumably due to only relatively small quantities of fusion protein being internalized in comparison to the amount injected. This result is in contrast to other studies, as at similar doses this fusion protein induces mortality via droplet feeding to larvae of *M. brassicae*,[6] another polyphagous pest of the same family as *L. oleracea* (Noctuidae). Differences in susceptibility may be due to variations in the target site of action of Hv1a, voltage-gated calcium channels,[25] or inability of the fusion protein to reach the CNS, where those channels are expressed. Whilst Hv1a/GNA was not orally toxic to *L. oleracea*, host larvae fed GNA, on the other hand, exhibited significant weight reduction, as previously reported,[26] thus demonstrating that the lectin was biologically active. It is not clear why the GNA on its own deleteriously affects the larval weight whereas the GNA-based fusion does not. It is possible that GNA being smaller in size is able to permeate the midgut more effectively than the

larger fusion protein; alternatively by attaching the Hv1a toxin to the N-terminus of the lectin inhibits the formation of the tetrametric molecule resulting in reduced binding of the GNA to gut receptors.

Exposure routes are a major consideration in the experimental design, as parasitoids can be exposed to the biopesticide in many different ways, particularly via its hosts. Therefore, in order to represent a field-relevant scenario, a tri-trophic system via host larvae was used, as it enabled an investigation as to whether ovipositing parasitoid females would avoid contaminated hosts and, if not, whether *E. pennicornis* larvae would be negatively affected by the recombinant proteins. Furthermore, if the fusion proteins were to be applied on the crops or expressed in transgenic plants, adult parasitoids would have minimal exposure, as they are unlikely to feed on plant parts other than pollen and nectar.[27]

The environmentally safe use of Hv1a/GNA as a biopesticide for the control of *M. brassicae* in Brassicaceae, tomatoes and a wide range of plants, which are also attacked by *L. oleracea*, should exclude any effect of the fusion protein on the pest's natural enemies, which play an important role in biological control. The use of a non-sensitive host, *L. oleracea*, provided an effective system to test direct effects of Hv1a/GNA on the parasitoid *E. pennicornis*, due to the fact that host quality, when considering size and weight, could be excluded as variables explaining potential differences between treatments. Furthermore, administering the fusion protein to parasitoids via hosts provides a realistic scenario, to some extent mimicking the route by which *E. pennicornis* would be exposed to Hv1a/GNA in crop systems. Although *L. oleracea* larval weight was affected by the GNA treatment, this

difference in host quality did not influence any of the parameters evaluated on the development of *E. pennicornis*. This is consistent with previous results with hosts feeding on GNA-containing diets. For example, Bell et al.[14] showed that maize-based and potato leaf-based diets containing GNA, and transgenic potato leaves expressing GNA fed to host *L. oleracea* did not have negative effects on *E. pennicornis*. Conversely, Wakefield et al.[15] reported a direct effect of GNA on *E. pennicornis* larvae, as none of the eggs deposited on GNA-fed or injected *L. oleracea* developed to the adult stage. The inconsistency between the present study and the results presented by Wakefield et al.[15] may be due to higher levels of GNA (50 µg/larvae) being injected into host larvae compared to that used in the present study. These lower levels may have influenced the ability to detect the GNA within the parasitoid larvae. However it can not be ruled out that these differences are due to different biological activities of the recombinant GNA used in the two studies.

The rate of parasitism of *E. pennicornis* adult females was not affected by treatment. Since Hv1a/GNA and GNA were present in *L. oleracea* haemolymph, it is reasonable to assume that parasitoid larvae that developed on those hosts were exposed to test proteins. However, attempts to detect the fusion protein in parasitoid larvae feeding on orally dosed hosts were not successful, possibly due to only low levels of fusion protein being present. To address this possibility, parasitized *L. oleracea* hosts were injected with high amounts (15 µg/larva) of Hv1a/GNA to ensure exposure of the larvae to the fusion protein and to facilitate Hv1a/GNA immuno-detection within the parasitoid larvae. Following western blot analysis of those parasitoid samples, none of the bands that reacted with anti-GNA antibodies presented the correct molecular weight of intact Hv1a/GNA (ca. 25 kDa). This result indicates that the fusion protein

was being degraded by *E. pennicornis* larvae, which might explain the lack of toxicity when parasitoids were exposed to orally dosed hosts. To address this possibility, and to ensure that neonate parasitoid larvae were exposed to intact Hv1a/GNA, it was necessary to inject host larvae post parasitism, but prior to egg hatch. Unfortunately this resulted in high levels of mortality in all treatments, presumably as a consequence of compromised immunity, particularly in the fusion protein treatment. Despite only a small number of parasitized *L. oleracea* surviving, it was still possible to demonstrate that *E. pennicornis* pupae were able to emerge in all treatments and that the presence of the fusion protein did not significantly affect any of the parasitoid parameters measured.

Regulation (EC) 1107/2009 and Directive 2009/128/EC[28] relating to the registration and sustainable use of pesticides within the EC require member States to reduce the risks and impacts of pesticide use on human health and the environment.[29] If proven to be effective in field trials, fusion proteins that target insect pests while being innocuous to non-target, beneficial arthropods provide a promising step towards novel environmentally friendly pest control strategies. Recent studies to investigate effects of this same biopesticide on another hymenopteran, the honey bee (*Apis mellifera*), demonstrated its safety at field-relevant doses in terms of contact, acute and chronic toxicity. Importantly, Hv1a/GNA was also shown to have no effect on bee behaviour (learning and memory), a critical consideration for pollinators.[30] From the experimental work carried out with honeybees and the parasitoid wasp, it is likely that hymenopteran voltage-gated calcium channels do not interact or interact poorly with Hv1a. Further research with other hymenopteran species are necessary in order to confirm this hypothesis. Results from the present study similarly demonstrate that

the fusion protein Hv1a/GNA does not affect important life history parameters of the parasitoid *E. pennicornis* and is thus unlikely to compromise this particular parasitoid as a biological control agent.

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Tables and Figures

Table 1: Comparison of the exposure of *E. pennicornis* larvae to hosts that ingested BSA (control), GNA or Hv1a/GNA.

	BSA	**GNA**	**Hv1a/GNA**
Mean number of pupae/host	26.25±3.62(16)[a]	23.72±3.35(11)[a]	25.5±3.62(14)[a]
Mean number of emergences/host	20±3.59(11)[a]	15.25±1.96(8)[a]	17.3±1.96(10)[a]
Sex ratio (males:females±SE)	0.18±0.03[a]	0.17±0.03[a]	0.11±0.02[a]
% emergence rate	65% (11)[a]	68% (8)[a]	71% (10)[a]

Same superscript level letters mean that there are no significant differences between treatments ($p>0.05$). Numbers in brackets represent the number of host larvae per analysis. % emergence rate calculated based on the number of viable pupae.

Table 2: Comparison of exposure of *E. pennicornis* larvae to hosts injected with 15 µg of BSA (control), GNA or Hv1a/GNA.

	BSA	**GNA**	**Hv1a/GNA**
Number of injected hosts	34	34	50
Surviving hosts 48h post injection	12	12	2
Mean number of pupae/host	3.8±1.5 (12)[a]	8.8±3.4 (12)[a]	2±2 (2)
Mean number of emergences/host	6.6±1.8 (6)[a]	12.5±4.8 (7)[a]	4 (1)
% emergence rate	91.6±8.3 (6)[a]	79±6.1 (7)[a]	100 (1)

Same superscript level letters mean that there are no significant differences between treatments (P>0.05).). Numbers in brackets represent the number of host larvae per analysis. As there were cases in which no parasitoid larvae developed to pupae, mean number of pupae per host appears lower than mean number of emergences per host. Due to low number of viable hosts, no comparisons were made between Hv1a/GNA and other treatments. % emergence rate calculated based on the number of viable pupae.

Figure legends

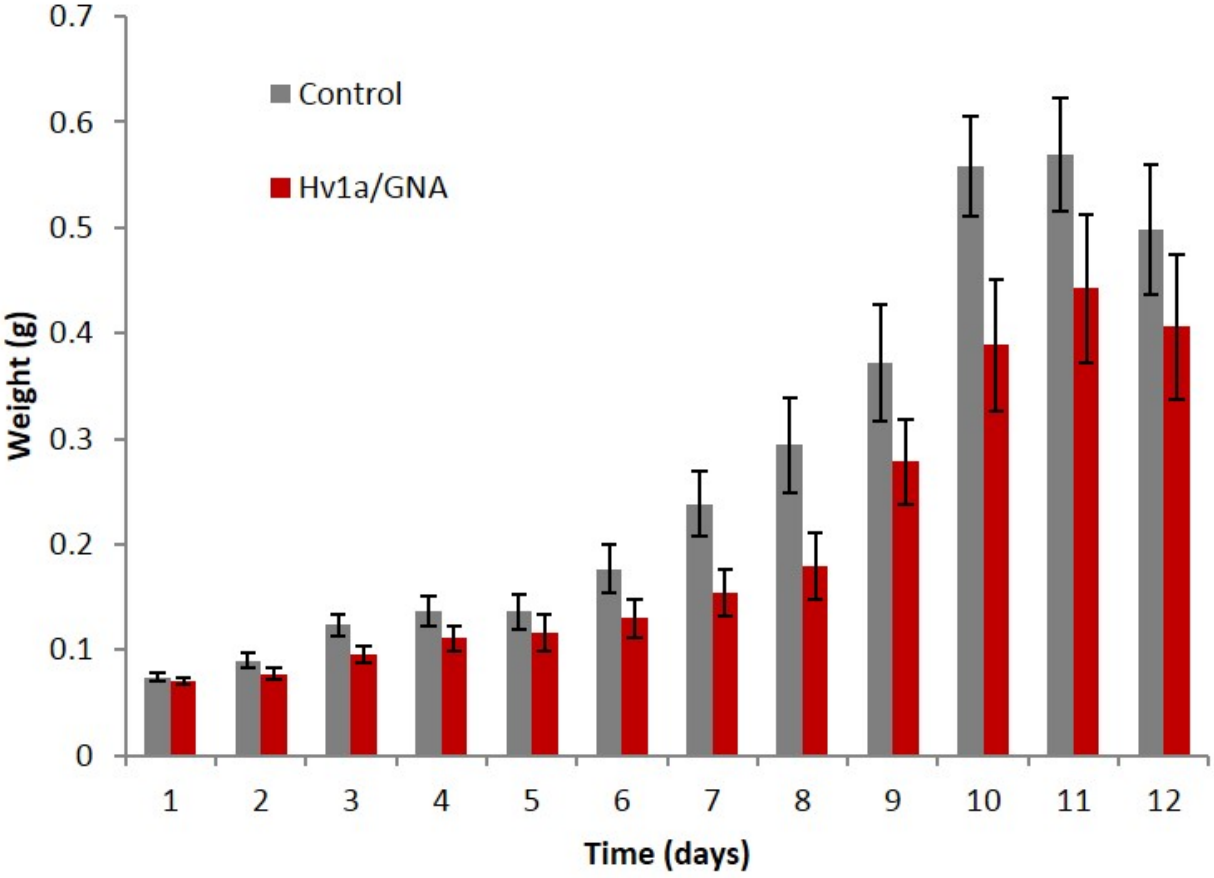
Figure 1: Effects of Hv1a/GNA (15 µg/larva) on *L. oleracea* via injection, compared with control (BSA) larvae. A significant reduction in mean weight (± SEM) was observed in the Hv1a/GNA treatment from day 2 to day 10 ($p < 0.05$). From day 11, there were no significant differences between treatments. Pairwise comparisons are significant at $p<0.05$.

Figure 2: Western blot showing internalization of Hv1a/GNA by *L. oleracea* larvae. 1) positive control (Hv1a/GNA); 2) haemolymph from larva fed with droplets containing GNA; 3) haemolymph from larva fed with droplets containing Hv1a/GNA. Intact Hv1a/GNA is indicated by the arrow; 4) Negative control (haemolymph from larva fed on droplets containing BSA).

Figure 3: Average weight (g) per day of 5th stage *L. oleracea*. The Hv1a/GNA treatment was not significantly different from the control treatment at any time point. From day 5 onwards, the GNA treatment was significantly different from the other treatments ($p < 0.05$).

Figure 4: Percentage of *E. pennicornis* parasitism on *L. oleracea*, per treatment. Difference between treatments is not significant ($p = 0.378$).

Figure 5: Hv1a/GNA is degraded following ingestion by *E. pennicornis*. Lanes: 1 and 2) positive controls (Hv1a/GNA and GNA, respectively), 3) Control (samples of parasitoid larvae feeding on hosts injected with BSA); 4) samples of parasitoid larvae feeding on hosts injected with the fusion protein, showing degradation of Hv1a/GNA.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0.3
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Weight (g)
Control
Hv1a/GNA
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3
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7
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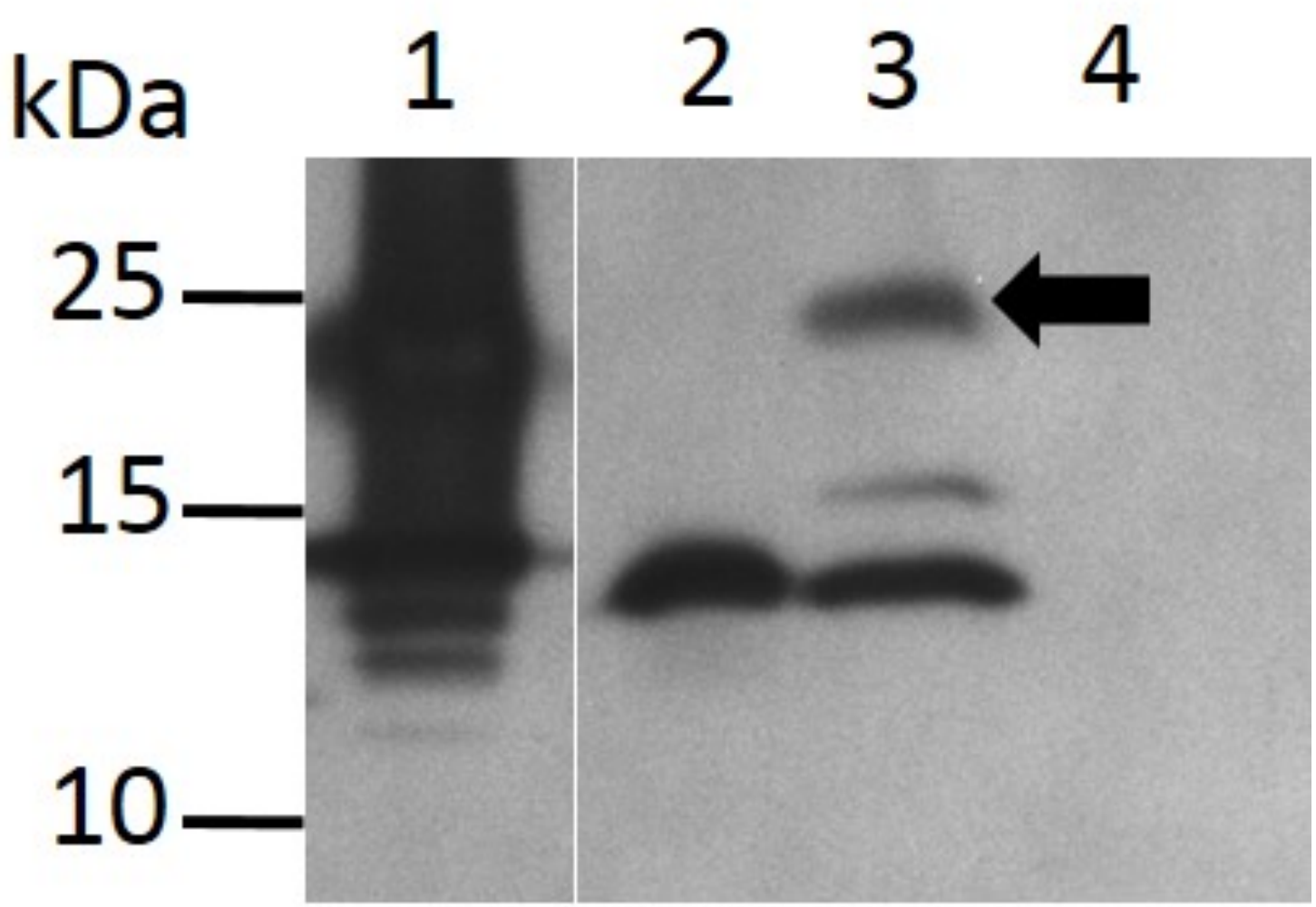
11
12
Time (days)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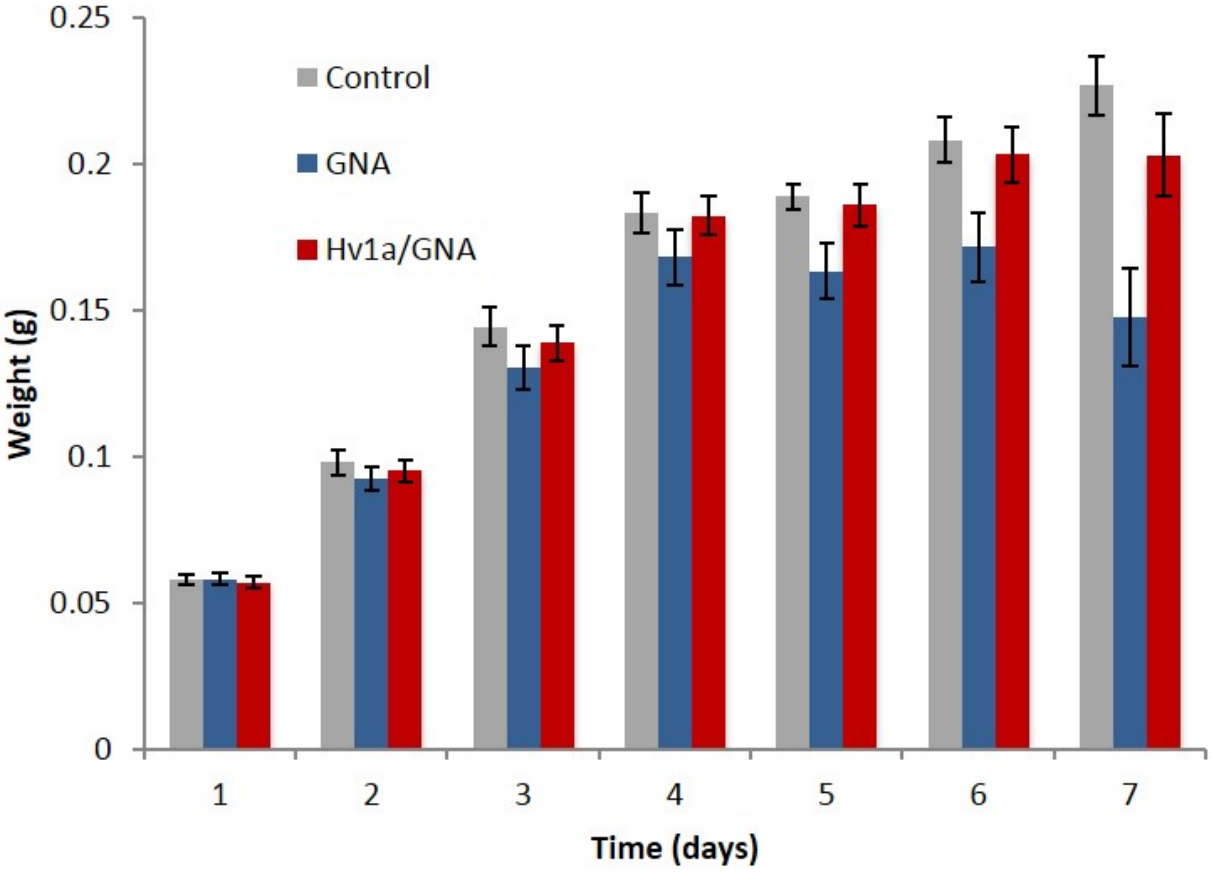
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Control
GNA
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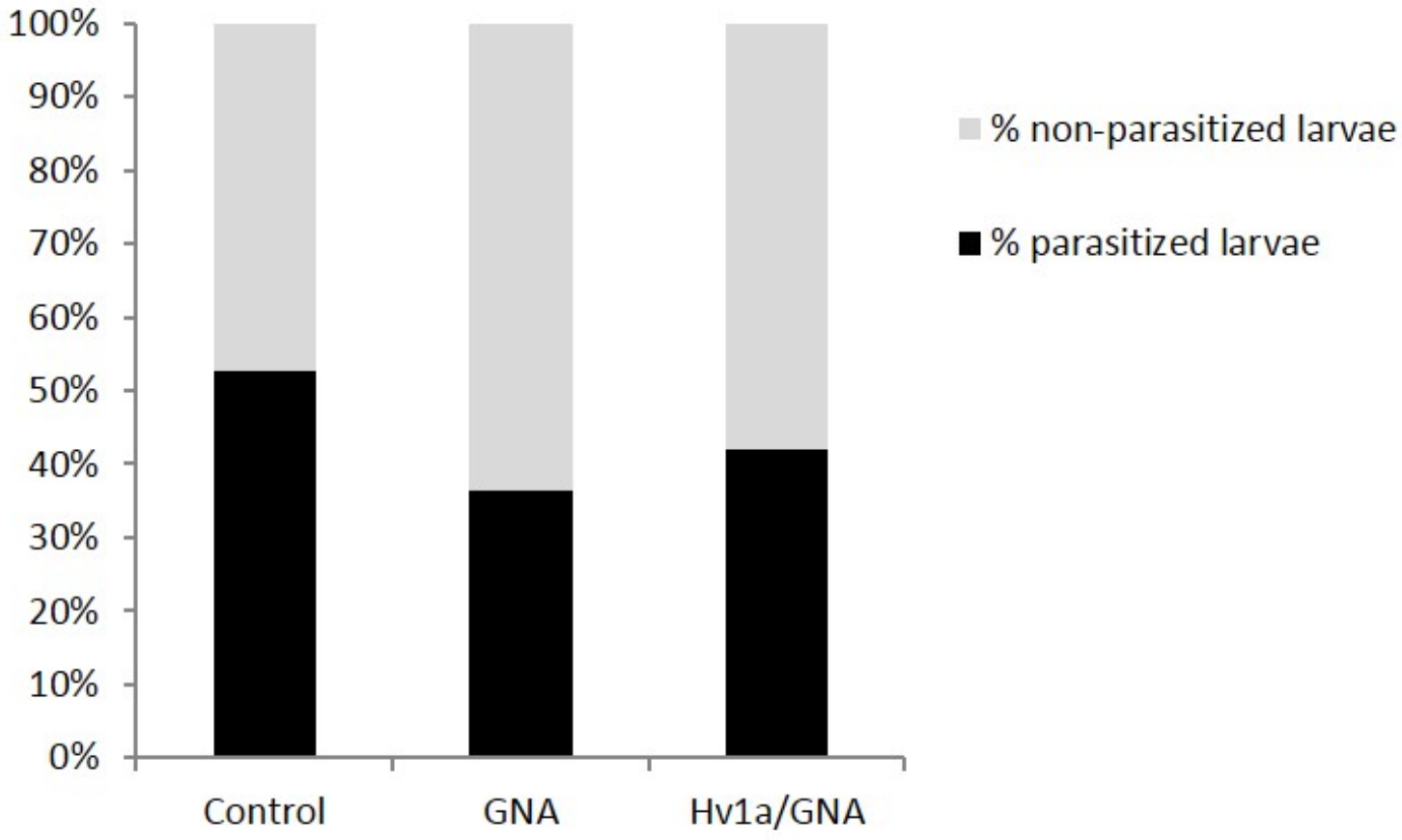
5
6
7
Time (days)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80%
70%
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Control
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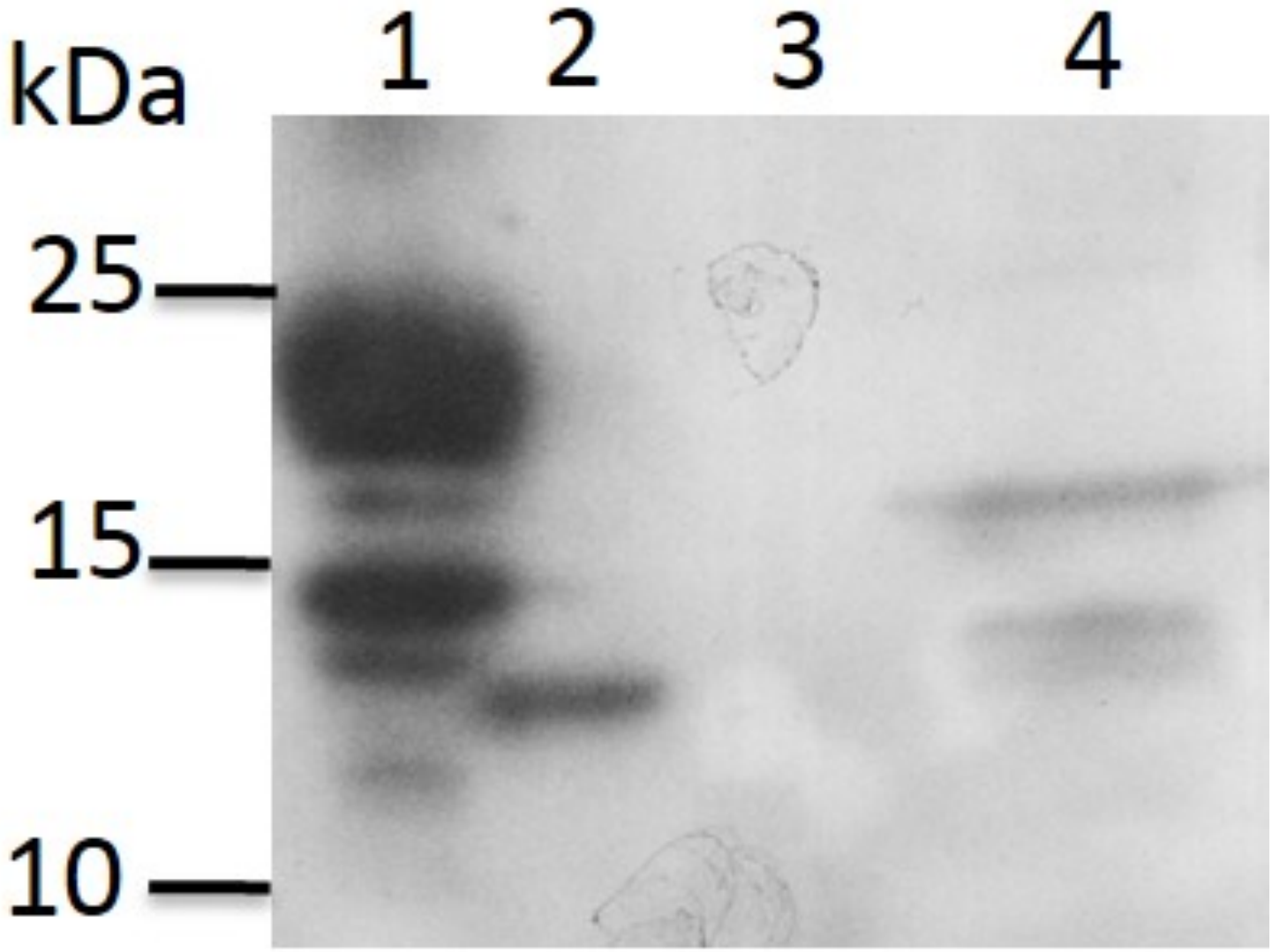
% parasitized larvae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